ENZYME ACTION IN FUCUS VESICULOSUS L.

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Little is known regarding the metabolism of the *Fucaceæ*. The chemical nature of the chief accumulation products has not yet been sufficiently investigated. In fact, prior to 1905 very little work of importance had been contributed on the products of any group of the marine algæ. Even the chemical determination of the carbohydrates, for example, in some of the larger groups of algæ, afforded no suggestion as to the nature of these products. More activity in this general field of work has been manifest, however, since the date referred to. Diverse views prevailed regarding the nature of the various granules which had been long detected microscopically. In the earlier literature Hansteen's ('92, '00) opinion has generally dominated, by which it was claimed that the granular bodies of the cell—and particularly the larger vesicular forms—contain fucosan, a carbohydrate, which was considered the first visible product of photosynthesis. On the other hand, Crato ('92, '93) maintained, from microchemical reactions, that the larger vesicles, physodes as he called them, contained phloroglucin, or some derivative of this body. Müther and Tollens ('04) found a methylpentosan in *Fucus* and *Laminaria*, while Koenig and Bettels ('05) among others found glucose and fructose, as well as pentoses and methyl pentoses, in *Laminaria* after hydrolysis. Swartz ('11) gives an extensive summary of the previous work on carbohydrate occurrence in the algæ, and contributes much data on the digestion of the hemicelluloses, but she studied no brown algæ. The existence of reducing sugars in *Fucaceæ* was clearly shown by Tihomirow ('10). Recently the carbohydrates have been more completely investigated by Kylin ('12, '13). Nevertheless, much remains to be done on these products, while

the proteins (aside from agar agar and related compounds) and other organic substances are scarcely known.

In view of the very considerable data on the carbohydrate metabolism in higher plants, it seems particularly desirable to investigate further this relation in the brown algæ. Moreover, no general study having been made, as far as we could learn, of the enzymes of the *Fucaceæ*, it seemed possible that a determination of the more characteristic enzymes, and of their distribution in *Fucus*, might lead to a better comprehension of the nature of the metabolism of these plants. Accordingly, during the summers of 1913–14 we have made an examination of *Fucus vesiculosus* with respect to its enzyme content.

In preparing the Fucus material for study we have followed several of the customary methods which have been found satisfactory in yielding enzymes of a high degree of efficiency. Since our results with *Fucus* have been so generally negative with respect to the presence of the commoner enzymes of plant metabolism, it may be well to indicate briefly how the material was handled. The Fucus plants were obtained in quantity, apparently in a condition of active growth, and the material was carefully picked over to avoid the contamination of attached animals and smaller algæ, then washed, and finally treated by one of several methods. Some of it was hung in a shaded, warm room until quite dry and brittle, then ground in a mill to an extremely fine powder, the latter being preserved in dry bottles for extraction, as subsequently indicated. For other phases of the work the plants fresh from the water were ground almost to a pulp in a meat grinder, sometimes passing the material twice or oftener through the machine. In some cases this fresh pulp, further comminuted in a mortar, or an extract from it, was used directly, while in other cases an alcohol-acetone dry preparation was made from it—the latter by treating alternately with 95 per cent alcohol (15 minutes) and acetone (5–10 minutes) until practically dehydrated, with a final brief treatment with absolute alcohol or ether, when the material was spread out on filter paper to dry. The alcohol-acetone material was thoroughly pulverized in a mortar for further use.

In the preparation of extracts the dry material was treated with distilled water (usually 10 parts of water to 1 part of

material), or in some cases with sea-water, using commonly 20 per cent alcohol or 2–3 per cent toluene as a preservative. In general, toluene has proved the most satisfactory antiseptic. The filtered extract was then precipitated with 95 per cent alcohol, the precipitate caught on a filter, washed with alcohol and dried. In a few cases the extract was used direct, and in certain respects the common practices were variously modified in the hope of detecting some simple explanation of the large number of negative results.

The hydrolytic experiments were carried out in small Erlenmeyer flasks or test-tubes, and always in duplicate or triplicate. In addition, nearly every series was repeated once or oftener. A special effort was made to determine the presence of carbohydrases, and for this purpose weak solutions, usually 0.5 per cent, of starch, glycogen, dextrin, saccharose, maltose, and lactose were employed in numerous tests. No reduction, or no change in the reducing value of the substrate, by the Fehling method, was found in any case in our final experiments, although in some cases a relatively large quantity of the supposedly enzyme-containing material was used. We found it necessary to purify the best dextrin obtainable by precipitation with 95 per cent alcohol from a strong aqueous solution. In the preliminary experiments, and chiefly with one preparation, traces of reduction were found with glycogen, but in many later experiments this finding was not confirmed.

Owing to the consistently negative results with these carbohydrates it seemed possible that there might be an adjustment of enzyme action in *Fucus* such that a relation of the mineral salts, as in sea water, might be requisite for highest action. Consequently the enzyme solution in one large series of experiments was diluted with double strength sea-water; in another case the material was extracted with sea-water; and finally, fresh material was used, making with it a diffusion in sea water. In every instance the result was negative.

Another possibility then suggested itself, namely, that the presence of certain inhibiting substances might account for the absence of hydrolytic change. Accordingly, the effect of the Fucus material on the activity of taka diastase was determined in this way: To 10 grams of ground fresh material 100 cc. of

water and 1 gram of taka diastase were added, this being permitted to stand for 5 hours, as in extraction, and the filtrate from this extraction was tested upon starch solution. The results were positive, indicating that no free substances were present which could inhibit diastase action. In another test 1000 grams of Fucus material were divided into two lots of 500 grams each. To one of these, 5 grams of commercial malt diastase were added, and both were then treated by the alcohol-acetone method, and subsequently extracted and precipitated in the usual way. The material to which diastase had been added gave positive tests for the hydrolysis of carbohydrates in an extensive series with dextrin, glycogen, saccharose, and laminarin; but a solution of the precipitate from the lot receiving no diastase produced no changes in these substrates. These experiments included controls of several kinds. With every substrate, boiled material was also used, and it is interesting to note that the "enzyme" material increased in reducing power with boiling.

The tests referred to in the previous paragraph seemed all the more important inasmuch as the Fucus material had been found to be strongly acid, and it seemed possible that this acidity alone might prove an injurious factor. From the experiments just mentioned it is seen, however, that acidity could scarcely have been an important consideration. A quantitative determination of the acidity was nevertheless made, by titration with NaOH, and it was found to be about .0565 N HCl. There is a slight increase in the acidity, if the pulp is permitted to remain in water 12 hours.

Owing to the determination by many, as, for example, Müther and Tollens ('04), Kylin ('13), Swartz ('11), and others of the presence of hemicelluloses, especially pentosans, in the marine algæ, and, further, since the commoner carbohydrate enzymes had not been identified by us, it seemed desirable to examine the material for pentosanase. The most available pentosan was that of cherry gum, accordingly this material in fresh condition was obtained and utilized in many tests with the Fucus preparation, the flasks being maintained at temperatures ranging from 27–40° C. Although the experiments were permitted to run for a period of several days, no reduction above the amount found

in the controls was obtained, and certainly no pentosanase active on this material could be assumed to occur abundantly in Fucus tissues.

Only one series of tests has been made to identify cellulase in the material here reported upon, and the results are presented with much reserve. Precipitated cellulose, prepared from filter paper, was employed, and the experiments were conducted at 40° C. The indications were that slight cellulase activity may occur.

By means of the action of the alcohol-acetone preparation upon a 4 per cent olive oil-casein emulsion, the lipolytic activity was investigated in the usual way. With the emulsion used alcohol is most serviceable as a preservative. In the tests referred to there was no indication of hydrolysis after one week; so the preparations were permitted to stand for two months, but still without change. That the conditions in the above case were otherwise favorable for lipolytic action is shown by the fact that the same substrate yielded with an alga of another family a decidedly positive test in two days. Several series of experiments were likewise carried out for the determination of esterases. With methyl acetate, ethyl acetate, and ethyl butyrate the Fucus material produced no change, irrespective of the concentrations employed.

In some of our preliminary experiments it had appeared that urease was present, but a careful investigation of this point demonstrated an error in the earlier results, and no amidases were discovered through the action upon 0.5 per cent solutions of urea, acetamid, methylamine, asparagin, diphenylamine, and acetanilid. In these experiments NH_3 determinations were made according to the method of Folin.

No liquefaction of gelatin or of agar occurred during a ten-day interval in a large series of test-tubes arranged with these two substrates. In the different tests these media were made neutral, alkaline, and slightly acid. In the neutral and slightly acid tubes no observable change occurred; but in those tubes containing a higher percentage of acid — both in those containing the Fucus preparation and in the controls — general liquefaction occurred. It is obvious, therefore, that these gel-forming proteins are not noticeably affected by any enzymes

occurring in the Fucus material. More extensive series of tests were arranged to determine the presence of proteinases which might act upon some more widely distributed native proteins, such as albumin, casein, and legumin. No tests were made to determine the transformation of these bodies into proteoses or peptones, but the formaldehyde method of determining amino acids was employed, and in no case had any transformation of these substances proceeded to the amino acid stage.

Glucose, levulose, and galactose were used in two series of experiments designed to determine the presence of zymase in the alcohol-acetone Fucus powder. No sufficient evidence, however, of the occurrence of this enzyme was obtained even when the most delicate tests were employed to determine the liberation of CO_2. The action of Fucus extract from the alcohol-acetone preparation upon tannin was tested by means of quadruplicate experiments. Two concentrations of tannin were used, 1 per cent and 2.5 per cent. The determinations were made by means of Jean's iodine method, but in no case did the flasks receiving the Fucus extract exhibit hydrolysis greater than that shown by the controls. Neither prepared nor fresh Fucus material gave sufficient evidence of oxidase or peroxidase action to be considered positive. Negative results were obtained both by the direct method with gum guaiacum, and by the indirect method, in which the reagent mentioned is used with hydrogen peroxide, and apparently acidity is not a determining factor. The use of benzidine seemed to indicate oxidase activity, but it has been clearly shown that the ease with which this reagent undergoes "spontaneous" oxidation in boiled solutions necessitates caution in using it as a test of oxidase activity. Tests for catalase by the usual method, evolution of oxygen on the addition of hydrogen peroxide, have clearly indicated that this enzyme is widespread in *Fucus*. It should be noted that the findings with respect to oxidase and catalase activity are in agreement with those of Atkins ('14). Catalase was very generally identified by him in the algæ, but evidence of oxidase in the *Fucaceæ* was obtained only with benzidine as a reagent.

The unexpectedly negative character of the experimental work here briefly outlined prompted us to make many repeti-

tions of experiments and minor modifications in technique not referred to in this preliminary account. The nature of the results, furthermore, made it seem desirable that a much more general study be made of the abundance and distribution of the enzymes in the various families of the marine algæ, and such an investigation is now in progress by one of us.

It would seem idle to attempt here an explanation of the negative results obtained, yet two or three possibilities have occurred to us which may be mentioned. The conditions of life of the *Fucaceæ*, especially the temperature relation, make it possible to suspect that metabolic changes occur at a very slow rate. If this is the case, it might be assumed that the commoner metabolic enzymes might be present in such small quantity that an indication of their presence would not be apparent by utilizing the methods ordinarily employed. The very fact that the capacity for food accumulation, that is to say, the "storage" of food materials, has not become highly developed in these forms suggests that the usual enzymes might not be found in abundance. Nevertheless, if such is the case, it may be pointed out that the present methods of enzyme work are very inadequate when applied to metabolic processes in general dealing with the transformation of products which do not accumulate in some quantity in the cell. In this connection attention may be drawn to Arber's ('01) observation on the slow rate of transformation of starch in the thallus of *Ulva latissima*, where a darkening period of from three to five weeks was required for the disappearance of this product.

The other possibility which has suggested itself is that in the cells of the *Fucaceæ* there may occur inhibiting substances which upon the death of the cell may form with the enzymes compounds from which the ferments cannot be again recovered. We have no evidence of the existence of any such bodies. Further investigation of *Fucus* and related algæ should perhaps throw some light upon the negative evidence produced by our extensive data.

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Literature Cited

Arber, E. A. N. ('01). On the effects of salts on the assimilation of carbon dioxide in Ulva latissima. Ann. Bot. **15**:39–69. 1901.

Atkins, W. R. G. ('14). Oxydases and their inhibitors in plant tissues. Part III: The localization of oxydases and catalase in some marine algæ. Scientif. Proc. Roy. Dublin Soc. N. S. **14**:199–206. 1914.

Crato, E. ('92). Die Physode, ein Organ des Zellenleibes. Ber. d. deut. bot. Ges. **10**:295–302. *pl. 18.* 1892.

———, ('93). Ueber die Hansteen'schen Fucosankörner. *Ibid.* **11**:235–241. 1893.

Hansteen, B. ('92). Studien zur Anatomie und Physiologie der Fucoideen. Jahrb. f. wiss. Bot. **24**:317–362. *pl. 7–10.* 1892.

———, ('00). Ueber das Fucosan als erstes scheinbares Product der Kohlensäure-assimilation bei den Fucoideen. *Ibid.* **35**:611–625. *pl. 14.* 1900.

Koenig and Bettels ('05). Die Kohlenhydrate der Meeresalgen und daraus hergestellter Erzeugnisse. Zeitschr. f. Unters. d. Nahrungs- u. Genussmittel **10**:457–473. 1905.

Kylin, H. ('12). Ueber die Inhaltskörper der Fucoideen. Arkiv f. Bot. **11**^{5}:1–26. *pl. 1.* 1912.

———, ('13). Zur Biochemie der Meeresalgen. Hoppe-Seyler's Zeitschr. f. physiol. Chem. **83**:171–197. 1913.

Müther and Tollens ('04). Über die Producte der Hydrolyze von Seetang (Fucus), Laminaria, und Carragheenmoos. Zeitschr. d. Ver. d. deut. Zucker Ind. **54**:59. 1904. [Cited by Swartz.]

Swartz, M. D. ('11). Nutrition investigations on the carbohydrates of lichens, algæ, and related substances. Trans. Conn. Acad. Arts and Sci. **16**:247–382. 1911.

Tihomirow, W. A. ('10). Sur la valeur de la réaction microchimique de la phénylhydrazine: pour la constatation du sucre dans les tissus des plantes. Ann. Jardin Bot. de Buitenzorg, Suppl. **3**^{2}:537–582. *pl. 13–15.* 1910.